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# The Human Mammary Gland Basement Membrane Is Integral to the Polarity of Luminal Epithelial Cells

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**We show that myoepithelial cell basement membrane derived E3 and E8 domains of laminin-1 are capable of polarizing luminal epithelial cells with regard to epithelial membrane antigen localization. This event is dependent on the $\alpha$6 integrin and results in aggregation and phosphorylation of the tyrosine residues of the focal adhesion kinase complex. We also demonstrate that uncultured normal luminal epithelial cells synthesize normal levels of $\beta$ and $\gamma$ laminin chains and reduced levels of $\alpha$ chains mRNA in common with malignant epithelial cells. In contrast normal myoepithelial cells synthesize all three constituent chains of laminin-1. Therefore in breast cancer the absence of myoepithelial cells could result in a lack of laminin $\alpha$ chains which may contribute to loss of polarity of malignant epithelial cells.** 

*Key Words:* **laminin; epithelial; polarity.**

## INTRODUCTION

The luminal epithelial cells of the human mammary gland are responsible for $\beta$-casein synthesis [1] whereas the basal epithelial (myoepithelial) cells have contractile properties [2, 3] and other functions which are as yet poorly understood. Approximately 20% of the luminal epithelial cells are in direct contact with the basement membrane and the remainder are adjacent to the myoepithelial cells. In the normal breast the epithelial cells are fully polarized as judged by apical secretion of $\beta$-casein [1]. In high-grade neoplasia, cells are rarely polarized and only a small proportion of breast cancer cells are in contact with myoepithelial cells.

The extracellular matrix (ECM) regulates the functional and morphological differentiation of murine and human mammary epithelial cells *in vitro* and *in vivo* [4, 5]. ECM also regulates growth and apoptosis and has a central role in the induction of tissue-specific gene expression [6–9]. It has been demonstrated that both physical and biochemical signal transduction are required for ECM-dependent tissue-specific gene expression [10]. The ECM component responsible for this is the E3 domain at the carboxy terminus of the $\alpha$1 chain of laminin-1 [11].

Laminins are a family of glycoproteins that form a unique cruciform structure and are made up of one heavy $\alpha$ chain (~400 kDa) and two light $\beta$ and $\gamma$ chains (130–200 kDa). To date 11 different isoforms have been identified. There are five different alpha chains [12–16] together with three beta [17–19] and two gamma chains [20, 21]. Laminin variants are expressed in a tissue-specific and developmentally regulated manner [22]. Major basement membranes contain at least one of the laminin variants. Laminins promote cell differentiation [23], adhesion [24], growth [25], and neurite outgrowth [26]. Laminin-1 contains two binding sites for heparin and heparan sulfate proteoglycan (HSPG) at the carboxy and amino termini of the $\alpha$1 chain [27].

Laminin has been shown to play a key role in invasion and metastasis of carcinomas [28, 29]. The peptide SIKVAV from the $\alpha$1 chain has been shown to induce the synthesis of matrix metalloproteinases [30] and to promote tumor growth [31]. The peptide YIGSR from the $\beta$1 chain has been shown to reduce tumor growth [32] and induce apoptosis [33].

In the breast, laminin is associated with the myoepithelial cells in normal and benign breast and in grade I infiltrating ductal carcinomas but absent in poorly differentiated carcinomas [2]. A characteristic of neoplastic mammary tumors is the loss of the myoepithelial cells and the basement membrane in the early stages [2, 34], although immunocytochemical evidence indicates that extracellular matrix proteins are still present around tumor cells [28]. The metastatic breast cell line MCF-7 has been shown to express the $\beta$1, $\beta$2, and $\gamma$1 chains but little or no $\alpha$1 and $\alpha$2 [35]. Soini *et al.* [36] demonstrated intense staining for the laminin $\beta$1 chain in stromal fibroblasts but in 5 of 15 cases the mRNA was found in the neoplastic cells. The $\gamma$2 chain was present in 11 of 16 carcinomas but found to be exclusively associated with the few remaining myoepithelial cells [37, 38]. Therefore from these studies it is apparent that in cancers, characterized by reduced

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numbers of myoepithelial cells, little or no $\alpha$1, $\alpha$2, and $\alpha$3 chains would be present.

In the normal human breast integrin complexes are predominantly present in the myoepithelial cells. The laminin-binding integrins ($\alpha$1, $\alpha$2, $\alpha$3, $\alpha$6, $\beta$1, and $\beta$4) are expressed weakly in the epithelial cells in a basolateral location whereas breast carcinoma cells showed variable losses, disordered expression, or downregulation of these subunits [39–41]. These changes have been shown to be associated with loss of polarity, tissue disorganization, increased aggressiveness, and metastasis [42–44]. One study showed that high expression of $\alpha$6 integrins correlated with reduced survival of 119 women with invasive breast carcinomas [45]. The important role of $\beta$1 integrins in cell-cell and cell-ECM polarity and differentiation has been demonstrated in renal cells [46]. It has been demonstrated that blocking the function of the $\beta$1 integrin in malignant breast cells resulted in reversion to a normal phenotype and blocking $\alpha$6 or $\beta$4 function in nonmalignant cells resulted in cells exhibiting a malignant phenotype [47].

In this study we confirm the expression pattern of laminin chains observed in the MCF-7 cell line [47] in normal luminal epithelial cells, and that this pattern is also seen in other breast cell lines. However, this expression pattern is shown to be normal for mammary epithelial cells and not a function of malignancy. The myoepithelial cells, however, do express both $\alpha$1 and $\alpha$2 chains of laminin. When breast epithelial cells are separated from their adjacent myoepithelial cells either physically or due to malignancy, the epithelial cells lose polarity which may be a consequence of the lack of laminin chains. We also demonstrate that polarity can be restored by culturing the luminal epithelial cells on either myoepithelial cells or laminin.

## MATERIALS AND METHODS

*Purification of primary cells.* Cells were purified from breast tissue from reduction mammoplasties using a procedure modified from a previous publication [48]. Briefly, breast tissue was cut into 0.5 $cm^3$ pieces and digested overnight at 37°C with collagenase (1 mg/ml) in RPMI 1640 plus 5% FCS. Following digestion the fat was decanted off and the remaining organoids and cells were washed three times in media. Following three 20-min sedimentation steps at 1 g (the supernatants were retained for stromal cell cultures), the organoids were washed three times with cold RPMI 1640 containing 1% FCS and digested with trypsin/EDTA (0.05%/0.02% in PBS) plus 0.4 mg/ml DNase for 15–30 min at 37°C, and the reaction was terminated by the addition of cold RPMI plus 10% FCS. Breast cells were separated using antibody-coated magnetic beads (Dynabeads, Dynal, UK Ltd., New Ferry, Wirral, UK), by incubating the preparation serially with anti-common acute lymphoblastic leukemia antigen (CALLA)-coated beads (as a marker for myoepithelial cells) until no more cells were isolated followed by incubation with anti-epithelial membrane antigen (EMA) antibody-coated beads (as a marker of epithelial cells) as previously described [48]. Each cell preparation was examined by RT-PCR for EMA and CALLA expression to ensure purity [48].

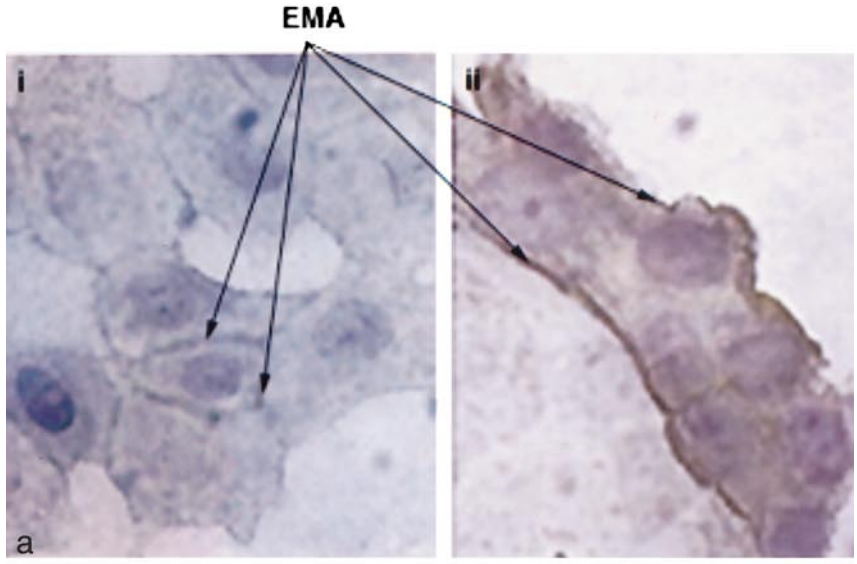
*Cell culture.* Myoepithelial cells were cultured in the serum-free mammary epithelial growth media (MEGM) (Clonetics Corp.) or CDM-5 (DMEM/F-12 containing, 10 nM Hepes, 10 nM L-glutamine, 100 U/ml penicillin, 200 $\mu$g/ml streptomycin, 50 U/ml polymixin B, 2.5 $\mu$g/ml amphotericin B, insulin 3 $\mu$g/ml, hydrocortisone 0.5 $\mu$g/ml, estradiol 1 nM, fetuin 20 $\mu$g/ml, holo-transferrin 25 $\mu$g/ml, phosphoethanolamine 0.1 mM, BSA 0.01%, ascorbic acid 10 $\mu$g/ml, dibutyryl-cAMP 10 nM, sodium selenate 2.6 ng/ml, EGF 100 ng/ml, triiodothyronine 10 nM, trace element mix 1 $\mu$l/ml, and fibronectin 100 ng/ml). The separated epithelial cells were cultured in BCM (DMEM/F-12 containing, 15 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 U/ml polymixin B, 2.5 $\mu$g/ml amphotericin B, 5 $\mu$g/ml insulin, 10 $\mu$g/ml apo-transferrin, 100 $\mu$M ethanolamine, 1 $\mu$g/ml hydrocortisone, 10 ng/ml EGF, and 10% FCS). The breast cell lines were all cultured in RPMI 1640 plus 10% FCS and 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin. The lung adenocarcinoma cell line HU-1 (a kind gift from Dr. Ulla Wewer, University Institute of Pathological Anatomy, Copenhagen) was cultured in Dulbecco's MEM plus 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and 10% FCS.

*EMA localization.* Coverslips were coated with laminin, fibronectin, and type IV collagen (Sigma, Poole, UK) at a concentration of 10 $\mu g/cm^2$, dried overnight, and washed in PBS containing 0.5% BSA. Coverslips were coated with Matrigel (Collaborative Research Inc.) at the same concentration, dried, and washed with serum-free media. Slides were coated with type I collagen (Collagen Corp., Palo Alto, CA) at 1 mg/ml and washed with PBS containing $Ca^{2+}$ and $Mg^{2+}$. The cells under investigation (HBr-SV-161, MDA-MB-231, MCF-7, and normal separated epithelial cells) were plated out onto the coverslips at $10^5$ cells/slide and cultured for 48 h. The cells were fixed in 4% Formalin, blocked with PBS containing 5% BSA and 10% normal goat sera. The anti-EMA antibody was used at a concentration of 5 $\mu$g/ml. The secondary antibody (anti-rat IgG, HRP conjugate was used at a dilution of 1:250) was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB). The cells were counterstained in Gill's hematoxylin, dehydrated, cleared, and mounted.

*Blocking experiments.* Each of the cell types was cultured for 48 h on laminin, type I collagen, and glass in the presence of anti-$\beta$1 antibody (Chemicon, Harrow, UK), anti-$\alpha$6 antibody (Chemicon) both at a concentration of 3 $\mu$g/ml [49] or mouse IgG as a negative control. The peptide KEGYKVRLDLNITLEFRTTSK from the laminin E3 domain has been shown to specifically block laminin-induced casein expression in mouse [11]. This peptide and a randomly scrambled version were added to the media at a concentration of 100 $\mu$g/ml to epithelial cells cultured on laminin and glass. After 2 days in culture the cells were stained for EMA using FITC as the secondary antibody.

*Immunofluoresecent staining for FAK and $PY_{20}$.* Epithelial cells were cultured for 48 h on glass or laminin and in the presence of the integrin $\alpha$6 blocking antibody or mouse IgG and stained for FAK (Upstate Biotechnology) and $PY_{20}$ (Santa Cruz). Each primary anti-

**FIG. 1.** EMA staining of mammary epithelial cells and cell lines. (a) The (i) nonpolar, (ii) polar staining of EMA of MCF-7 cells cultured on glass and laminin, respectively. In (b) The cells were cultured on glass and types I and IV collagen and in (c) on fibronectin, laminin, and Matrigel. The cells were stained for EMA and expression of this protein was graded on the basis of the following. EMA expressed on all cell membranes, =0, EMA on more than one cell membrane =+, EMA expressed on the apical membrane only =++.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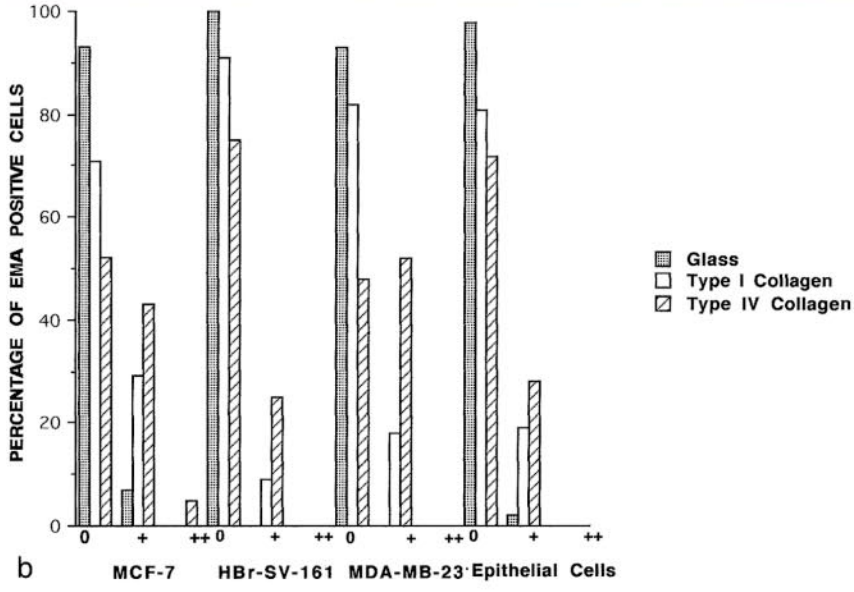
PERCENTAGE OF EMA POSITIVE CELLS
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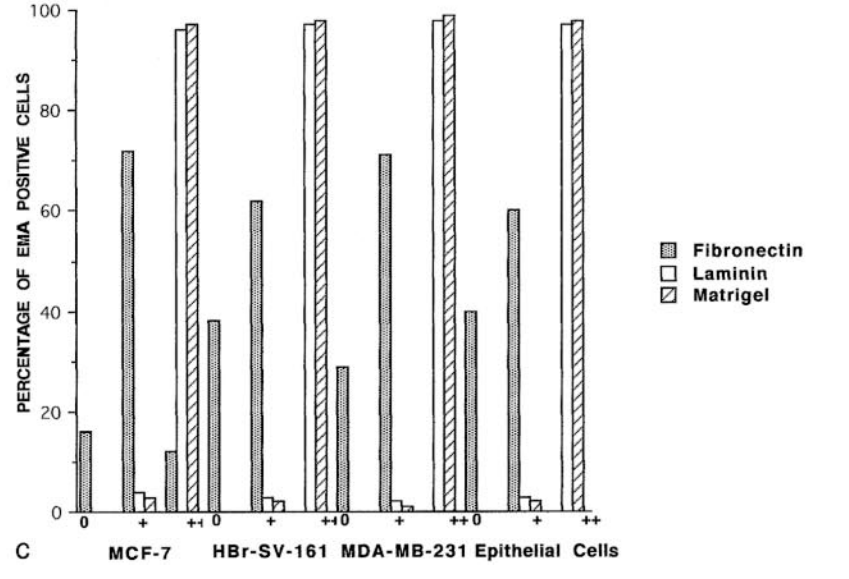
Glass
Type I Collagen
Type IV Collagen
b


PERCENTAGE OF EMA POSITIVE CELLS
100
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60
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MCF-7 HBr-SV-161 MDA-MB-231 Epithelial Cells
Fibronectin
Laminin
Matrigel
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body was used at a concentration of 5 $\mu$g/ml using FITC and TRITC as the secondary antibodies, respectively.

*RT-PCR.* Total RNA was isolated using RNAzol (Biogenesis) according to the manufacturers instructions. A 20-$\mu$l reverse transcriptase reaction contained 2 $\mu$g total RNA, 300 $\mu$g of random primers, 4 $\mu$l of 5X RT buffer (Gibco BRL, Paisley, UK), 2 $\mu$l of 0.1 M DTT, 1 $\mu$l of 25 mM dNTPs, 1 $\mu$l $H_2O$, and 1 $\mu$l of reverse transcriptase (Gibco BRL, Paisley, UK). The primers and conditions for the $\alpha$1 and $\alpha$2 chain were according to Engvall *et al.* [50]. For laminin $\beta$1 the cDNA was amplified using 30 cycles consisting of (1) denaturing for 60 s at 94°C, (2) annealing primers for 60 s at 52.7°C, and (3) extending primers for 120 s at 72.5°C. The primers were GCCATCATCATGTC-CTGGTCAG and ACGGCGTTGCCATAGTAG; the calculated size of the PCR product is 259 bp. For laminin $\gamma$1 the cDNA was amplified using 30 cycles consisting of (1) denaturing for 60 s at 94°C, (2) annealing primers for 60 s at 54°C, and (3) extending primers for 120 s at 72.5°C. The primers used were GATGGCTGTGGATCTTTG and ATGACAGTGCTGTCTGGAC; the calculated size of the PCR product is 165 bp. Each of the products was analyzed on 1.5% acrylamide gel. Control amplification of RNA samples without prior cDNA synthesis did not detect contaminating genomic DNA as judged by the absence of ethidium bromide-stainable PCR product. We used cDNA for each of the respective chains (kind gifts from Prof. Karl Tryggvason, Biocenter, Oulu, Finland) as positive controls.

*Immunohistochemistry.* Frozen sections of 8 $\mu$m from normal and malignant breast tissue were stained using an indirect peroxidase method. Sections were fixed in 4% Formalin, blocked with 5% BSA in PBS plus 10% goat sera, and stained for laminin $\alpha$1, $\alpha$2, $\beta$1, and $\gamma$1. The primary antibodies (Chemicon) were used at 5 $\mu$g/ml for $\alpha$1, $\beta$1, and $\gamma$1 and at 10 $\mu$g/ml for $\alpha$2. Secondary antibody (HRP conjugated anti-mouse IgG [Sigma, Poole, UK]) was used at a dilution of 1:250. The sections were developed in DAB and counterstained in Gill's hemotoxylin.

*Western blotting.* Cultured cells were rinsed with PBS, scraped off, and extracted in boiling sample buffer. Each preparation was then sonicated to reduce viscosity and concentrated fivefold using Centricon 30 (Amicon). Purified EHS laminin ($\alpha$1-$\beta$1-$\gamma$1) was Coomasie blue-stained and used as 400 and 200 kDa molecular weight markers. SDS-PAGE was performed according to Laemmli using 4–8% linear gradient gels and a 3% stacking gel. Samples were transferred to nitrocellulose and then blocked in 3% nonfat dry milk in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl for 60 min at room temperature. Primary antibodies were diluted to 5 $\mu$g/ml for $\beta$1 and $\gamma$1 and 10 $\mu$g/ml for $\alpha$2 in blocking buffer for 60 min at room temperature. Secondary antibodies (goat anti-mouse IgG conjugated with horseradish peroxidase) were diluted in blocking buffer and incubated for 60 min at room temperature. The HRP was detected using the enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturers specifications.

## RESULTS

The polarity of epithelial cells is affected by the extracellular matrix [6–9]. Since few mammary epithelial cells are in direct contact with the basement membrane we investigated whether the polarity of these cells may be affected by the adjoining myoepithelial cells and hence indirectly by the myoepithelial cell basement membrane.

We have recently demonstrated that EMA is localized to a single membrane when luminal epithelial cells are cultured in the presence of myoepithelial cells embedded in Matrigel and formed bilayered ductal structures [51]. In order to investigate whether a constituent of the Matrigel was responsible for this effect we cultured the malignant cell lines MCF-7 and MDA-MB-231, the normal epithelial cell line HBr-SV-161 (Dr. M. O'Hare, Ludwig Cancer Institute), and normal primary epithelial cells on glass and thin layers of type I collagen, on thin layers of the extracellular matrix components fibronectin, type IV collagen, and laminin, and on Matrigel. After 48 h in culture they were stained for EMA. An example of this is shown in Fig. 1a where we have demonstrated cells (left panel) judged to be nonpolar, since EMA staining is seen around the entire cell. The right panel shows two opposed cell layers each with EMA staining on one surface only and judged therefore to be polar with respect to EMA staining. The staining observed appears to show inverse polarization of the epithelial cells and this is in agreement with a previously published study using breast-carcinoma-derived epithelial cells [52]. In EMA-expressing cells we observed that on glass, type I collagen and type IV collagen EMA staining was not restricted to a single membrane (Fig. 1b) but was predominantly localized to all the cell membranes. In the majority of cells (60–75%) cultured on fibronectin EMA staining was only evident on two of the cell membranes; however, on laminin and Matrigel virtually 100% of EMA staining was restricted to a single membrane (Fig. 1c). The cell lines and the primary epithelial cells were cultured in the presence of 10% FCS which contains both fibronectin and vitronectin. In view of previously published data [53, 54], we were surprised that the MCF-7 cells only polarized when cultured on laminin; however, these studies taken together may purely demonstrate the heterogeneity of cell lines.

When the primary luminal epithelial cells were cultured on laminin and Matrigel in the presence of mouse IgG, EMA staining was confined to a single cell membrane (Fig. 2A). EMA is localized on the membrane that borders the lumen *in vivo* and *in vitro* and the EMA is localized to the membrane that borders the surrounding milieu. In the presence of anti-$\alpha$6 integrin antibody EMA staining was dispersed (Fig. 2B), indicating that the polarizing effect of laminin was mediated by the $\alpha$6 integrin and that no other component of Matrigel was involved. In the presence of an anti-$\beta$1 blocking antibody the primary cells were rounded and only weakly attached to the substrate. EMA staining on the other substrata was unaffected by the presence of either antibody (data not shown). We also performed these experiments with MCF-7, MDA-MB-231, and HBr-SV-161 cell lines and these gave comparable staining for EMA.

When the scrambled peptide was added to the culture media of the primary luminal epithelial cells, EMA staining was confined to a single membrane (Fig. 2C), but in the presence of the E3-derived peptide

(KEGYKVRLDLNITLEFRTTSK) EMA staining was evident on all the cell membranes (Fig. 2D). The cells plated out on laminin in the presence of either IgG or the scrambled peptide reached confluence within 48 h but not those cultured in the presence of the anti-$\alpha$6 integrin antibody or the E3-derived peptide. Therefore since the same number of cells were plated out, the undifferentiated cells had a faster growth rate than the differentiated cells.

Focal adhesion kinase is known to aggregate in focal contacts in the presence of ECM components but there was no aggregation of FAK or phosphorylation of the tyrosine residues in the epithelial cells cultured on glass irrespective of the antibody present (data not shown). When the epithelial cells were cultured on laminin in the presence of the mouse IgG antibody or the scrambled peptide there was aggregation of FAK in the focal adhesions (Fig. 3A) and there was phosphorylation of the tyrosine residues of the FAK complex (Fig. 3B). However, in the presence of the $\alpha$6 integrin blocking antibody or the E3-derived peptide there was no aggregation of FAK (Fig. 3C) or phosphorylation of the tyrosine residues (Fig. 3D).

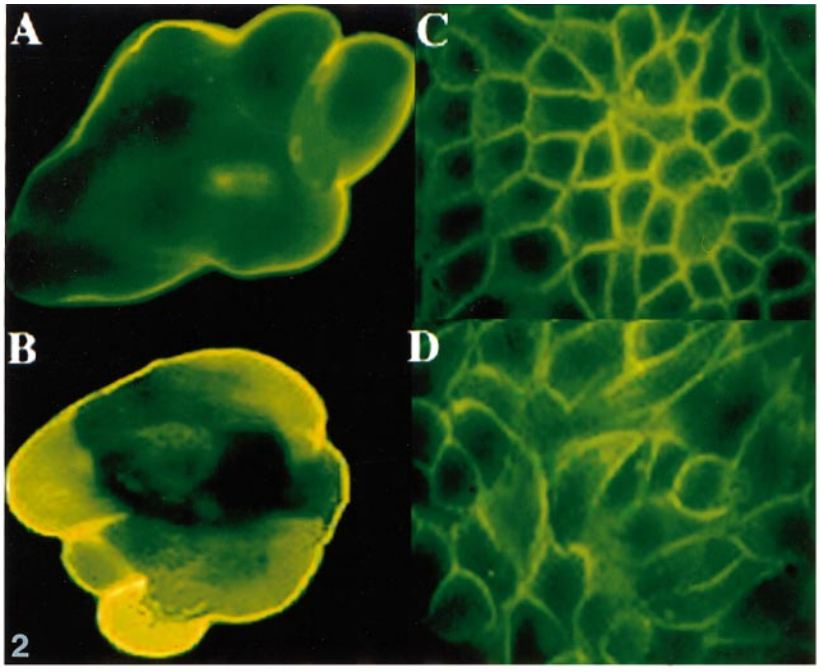
Wewer *et al*. [35] demonstrated that the malignant mammary cell line MCF-7 expressed negligible amounts of the laminin heavy chains $\alpha$1 and $\alpha$2; however, there was high expression of the light chains $\beta$1, $\beta$2, and $\gamma$1. As laminin synthesis has been suggested to be an artefact of tissue culture [38] RT-PCR was performed before and after culture of separated normal mammary epithelial and myoepithelial cells. We examined laminin chain expression in the malignant epithelial cell lines MCF-7, T47D, and MDA-MB-231, together with the nonmalignant cell lines HBr-SV-161 and HBL-100. Purified uncultured epithelial cells from three patients were shown to express low levels of laminin $\alpha$1 (Fig. 4A, Lane 3). When the luminal epithelial cells were cultured there was an increase in the expression level of the $\alpha$1 chain. (Fig. 4A, lane 4). However, the myoepithelial cells expressed high levels of the $\alpha$1 chain both before and after culture (Fig. 4A, lanes 5 and 7). The $\beta$1 and $\gamma$1 chains were expressed at comparable levels by both cell type irrespective of culturing (data not shown). To ensure similar loading and purity we examined each preparation for actin expression (Fig. 4B) and for EMA and CALLA expression (Fig. 4C). Due to alternative mRNA splicing, four EMA PCR products can be generated [55].

All the breast cell lines expressed high levels of $\alpha$1 and $\beta$1 (Figs. 4D and 4E, lanes 5–7 and 8 and 9); however, the $\gamma$1 chain was reduced in the MDA-MB-231 and MCF-7 malignant cell lines (Fig. 4F, lanes 7 and 9). We were unable to detect the $\alpha$2 chain by RT-PCR in any of the cells including the myoepithelial cells; however, we were able to detect the cDNA positive control; therefore this was not due to the PCR conditions. HBL-100 (Dr. M. O'Hare), which we find possess many features of myoepithelial cells, such as CALLA staining, synthesized all the chains of laminin-1 in approximately equimolar quantities (Figs. 4D–F, lane 5).

We stained breast sections from normal and malignant tissue together with tissue from fibroadenomas and ductal carcinomas *in situ* (DCIS) for $\alpha$1, $\alpha$2, $\beta$1, and $\gamma$1 laminin chains. The $\alpha$1 and $\beta$1 chains were found in the myoepithelial cell basement membrane and not associated with the stroma in the normal sections (Figs. 5A and 5I). e also observed weak staining for the $\alpha$2 chain in normal sections (Fig. 5E). This was unexpected in view of the absence of $\alpha$2-chain mRNA by RT-PCR in the separated cells. In DCIS the expression of the $\alpha$1, $\beta$1, and $\alpha$2 chains became disordered and there appeared to be a failure to deposit the laminin chains into an ordered matrix (Figs. 5B, 5F, and 5J). In the neoplastic sections we observed weak and disordered expression of the $\alpha$1 and $\beta$1 chains and of the $\alpha$2 chain (Figs. 5C, 5G, and 5K). The staining for $\gamma$1 was identical to that seen with $\beta$1 (data not shown). All of the negative control slides were devoid of any staining (Figs. 5D, 5H, and 5L).

In view of the apparent discrepancy between RT-PCR and immunohistochemistry for laminin $\alpha$2, uncultured and cultured cells were examined by Western blotting in order to investigate whether the $\alpha$2 chain was present in the normal cells. These blots were also probed for the $\beta$1 and $\gamma$1 chains. Figure 6A indicates that cultured myoepithelial cells and to a lesser extent epithelial cells do synthesize $\alpha$2 but at a much reduced level as compared to the HU-1 cell line [35]. The blots probed for $\beta$1 (Fig. 6B) and $\gamma$1 (Fig. 6C) indicate that these chains are being secreted by all of the cells investigated, although the myoepithelial cells exhibit a more pronounced band as compared to the epithelial cells. We were unable to detect any of the constituent chains of laminin in the uncultured cells, although this may be due to the relative insensitivity of Western

**FIG. 2.** EMA staining of primary human mammary epithelial cells. (A) Cultured on laminin in the presence of mouse IgG showing EMA staining restricted to a single membrane. (B) Cultured on laminin in the presence of anti-$\alpha$6 integrin showing dispersed EMA staining. Each antibody was added at 2 $\mu$g/ml. Each experiment was repeated three times. EMA staining of mammary epithelial cells. (C) Cells cultured on laminin in the presence of scrambled peptide showing polar EMA staining comparable to Fig. 3A. (D) Cells cultured on laminin in the presence of blocking peptide showing dispersed EMA staining comparable to Fig. 3B (each peptide was used at 100 $\mu$g/ml). Each experiment was repeated twice. All photographs at a magnification $\times$500.



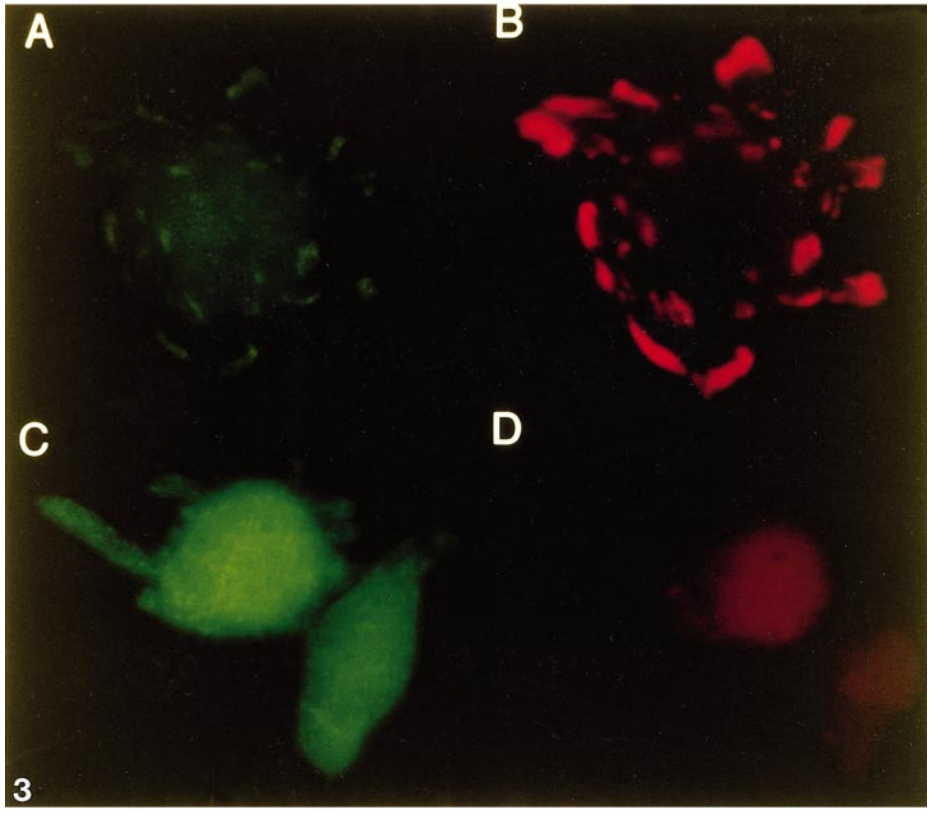
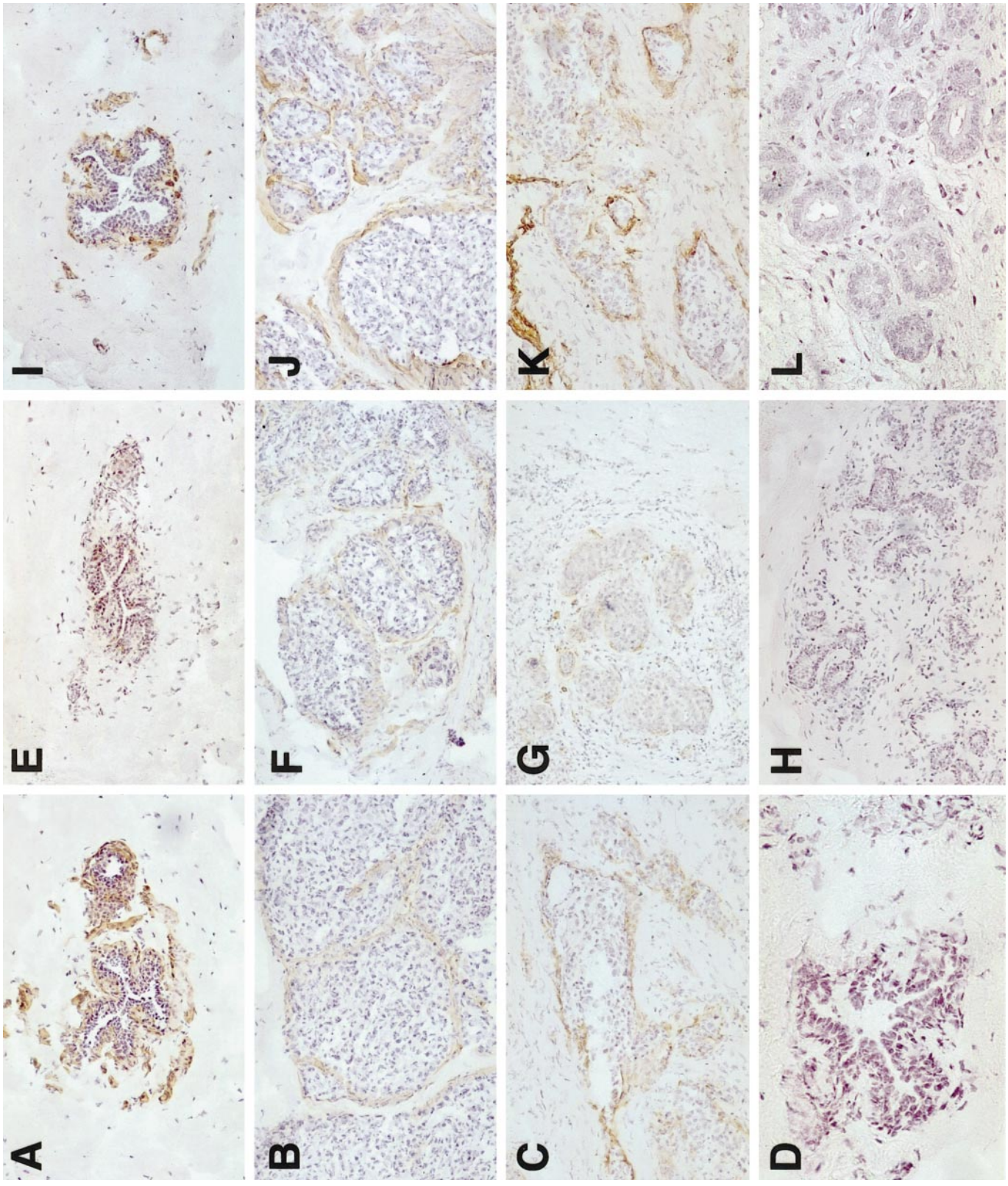




**FIG. 3.** (A) FAK staining of mammary epithelial cells cultured on laminin in the presence of mouse IgG and (B) PY20 staining showing accumulation of FAK at the focal contacts and phosphorylation of the tyrosine residues. (C) FAK staining of mammary epithelial cells cultured on laminin in the presence of $\alpha$6 integrin blocking antibody and (D) PY20 staining showing no aggregation of FAK or phosphorylation of the tyrosine residues. Each experiment was repeated with cells from a further two patients. All photographs at a magnification $\times$500.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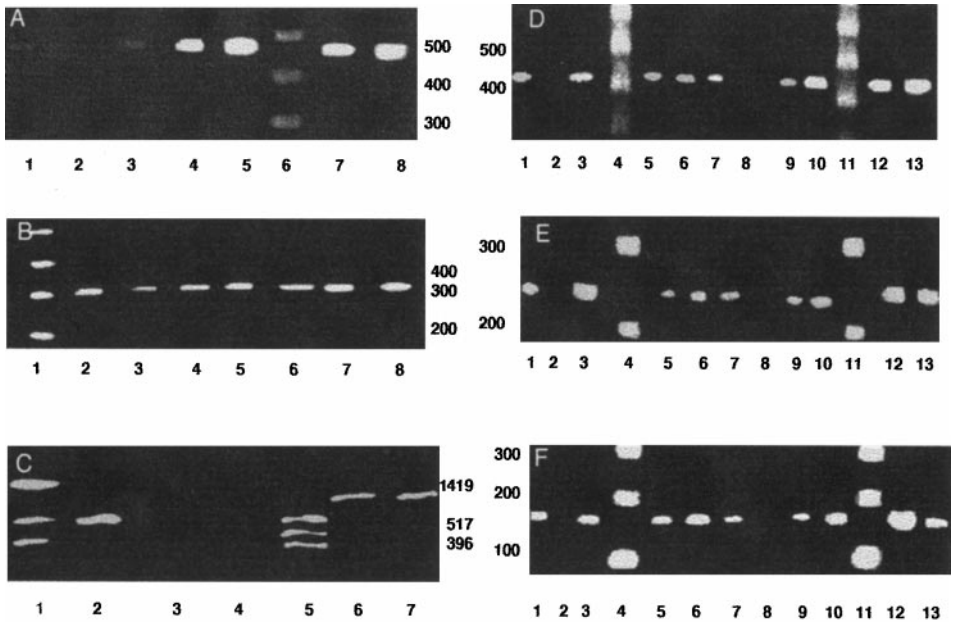


**FIG. 4.** Acrylamide gels of RT-PCR products. (A) Laminin $\alpha$1: Lane 1, MCF-7; Lane 2, cultured normal fibroblasts; Lane 3, uncultured normal epithelial cells; Lane 4, cultured normal epithelial cells; Lane 5, cultured normal myoepithelial cells; Lane 6, 100-bp ladder; Lane 7, uncultured normal myoepithelial cells; Lane 8, cDNA control. (B) Actin: Lane 1, 100-bp ladder; Lane 2, MCF-7; Lane 3, cultured normal fibroblasts; Lane 4, uncultured normal epithelial cells; Lane 5, cultured normal epithelial cells; Lane 6, cultured normal myoepithelial cells; Lane 7, uncultured normal myoepithelial cells. (C) Lane 1, *Hin*f1 restriction digest of pUC19 DNA; Lane 2, CALLA expression in myoepithelial cells; Lane 3, CALLA expression in epithelial cells; Lane 4, EMA expression in myoepithelial cells; Lane 5, EMA expression in epithelial cells; Lane 6, $\beta$-actin expression in myoepithelial cells; Lane 7, $\beta$-actin expression in epithelial cells. (D) Laminin $\alpha$1, (E) Laminin $\beta$1, and (F) Laminin $\gamma$1: Lane 1, cultured normal epithelial cells; Lane 2, cultured normal fibroblasts; Lane 3, cultured normal myoepithelial cells; Lane 4, 100-bp ladder; Lane 5, HBr-SV-161; Lane 6, T47D; Lane 7, MDA-MB-231; Lane 8, negative control; Lane 9, MCF-7; Lane 10, HBL-100; Lane 11, 100-bp ladder; Lane 12, cDNA positive control; Lane 13, HU-1 positive control. Each experiment was repeated with cells from a further two patients.

blotting and due to the fact that prior to culturing the cell numbers are low.

## DISCUSSION

In this paper we have examined the possibility that it is the presence of myoepithelial cells and their basement membrane that is responsible for polarizing luminal epithelial cells in the breast. This implies that in neoplasia where there is a reduction in the number of myoepithelial cells, the luminal cells lose polarity and this event may contribute to the neoplastic phenotype. We have found that laminin is capable of polarizing epithelial membrane antigen in both malignant and normal mammary epithelial cells and this effect is mediated by the $\alpha$6 integrin. We also found that all the primary luminal epithelial cells and the epithelial cell lines synthesize the $\beta$1 and $\gamma$1 chains but none of these cells synthesize significant amounts of the $\alpha$ chains. The fact that laminin $\alpha$1 chain is synthesized in culture but the cells are still in a nonpolarized state suggests that there is a defect in laminin assembly, deposition, or glycosylation. Myoepithelial cells are capable of synthesizing and depositing all three laminin-1 chains, *in vivo* and *in vitro.* These studies and others [35] have demonstrated that malignant cell lines synthesize minimal amounts of $\alpha$1 and $\alpha$2 chain *in vitro* but neither chain (nor $\alpha$3) is evident *in vivo* [38, 56], whereas $\beta$1 and $\gamma$1 were present at low levels [55]. Our data support these findings and taken together support the hypothesis that the absence of myoepithelial cells and their associated laminin $\alpha$ chains is responsible for the lack of polarity seen in malignant epithelial cells. The low amount of $\alpha$1 laminin mRNA observed in malignant epithelial cell lines may not be representative of

**FIG. 5.** Immunohistochemical staining of breast tissue for laminin chains. Laminin $\alpha$1, in (A) normal tissue, (B) ductal carcinoma *in situ,* (C) cancer, and (D) Negative controls. Laminin $\alpha$2 in (E) normal tissue, (F) ductal carcinoma *in situ,* (G) cancer, and (H) negative controls. Laminin $\beta$1 in (I) normal tissue, (J) ductal carcinoma *in situ,* (K) cancer, and (L) negative controls. (All photographs are at a magnification $\times$200.)

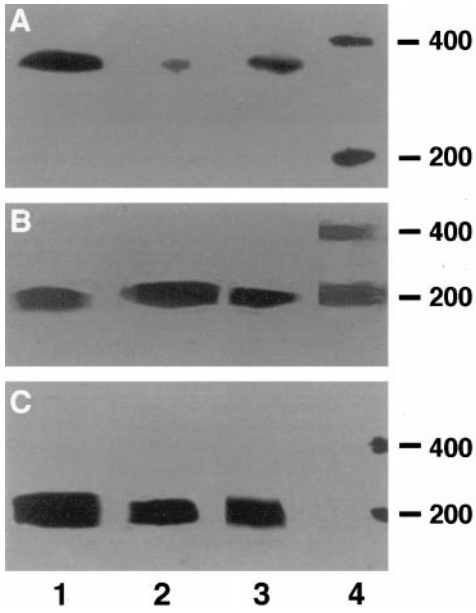


**FIG. 6.** Western blots of laminin chains. (A) Laminin $\alpha 2$, (B) laminin $\beta 1$, and (C) laminin $\gamma 1$: Lane 1, HU-1 cell line; Lane 2, cultured normal epithelial cells; Lane 3, cultured normal myoepithelial cells; Lane 4, Coomassie blue-stained EHS laminin as a molecular weight marker.

neoplastic epithelial cells *in vivo* but may reflect upregulation of laminin $\alpha 1$ on plastic as we observed with the separated epithelial cells. This suggests that *in vivo* normal human mammary epithelial cells rely on their associated myoepithelial cells for the synthesis of the $\alpha$ chain(s). Consequently as the myoepithelial cells are lost during carcinogenesis the epithelial cells no longer have access to the polarizing capabilities of the laminin $\alpha$ chain. The absence of the basement membrane therefore may mean that the malignant epithelial cells can more readily metastasize not only due to the absence of a physical barrier but also due to the undifferentiated state of the cells and the weakening of cell-cell adhesion.

It has been demonstrated that the $\alpha$ chain of laminin-1 drives secretion of the associated $\beta$ and $\gamma$ chains [57]. If this is the case in the human breast, and if there are no other $\alpha$ chains present, in the absence of myoepithelial cells no laminins will be secreted, possibly accounting for the failure to detect laminin in cancer [2]. However, if these unassembled chains are secreted, this may promote aberrant cell behavior, since the $\beta 1$ chain contains peptides capable of (a) binding to an elastin receptor with homology to the 67 kDa laminin binding protein [58], (b) promoting cell adhesion of melanoma cells [59], and (c) promoting melanoma cell migration and attachment [60].

It has been reported [42] that the absence of laminin in breast carcinomas is associated with reduced expression of $\alpha 6\beta 4$ integrin. However, since $\alpha 6\beta 4$ is a major component of the hemidesmosomes and luminal epithelial cells do not form hemidesmosomes even when in contact with the basement membrane (unpublished data), $\alpha 6\beta 4$ is probably specific to the myoepithelial cells, thus explaining its reduction in cancer. Experiments with a breast cancer cell line (MDA-MB-435) that had $\alpha 6\beta 1$ function knocked out by transfection with a dominant-negative deletion mutant of the $\beta 4$ integrin had a much reduced metastatic potential [61]. Taken together these data indicate that $\alpha 6$ may be capable of acting only as a tumor suppressor gene [62] when it forms a heterodimer with $\beta 4$ and that when it heterodimerizes with $\beta 1$ it may act to promote tumor progression. The reversion of malignant cells to a normal phenotype when the function of $\beta 1$ is blocked in cells that overexpress $\beta 1$ integrin [47] supports this hypothesis. The main conclusion that can be drawn from these series of experiments and ours is that it is not possible to extrapolate from one system to another. The reduction in $\beta 4$ expression in breast cancer means that the $\alpha 6$ chain will associate more with the $\beta 1$ subunit. Our data indicate that the $\alpha 6\beta 1$ integrin will have little or no ligand in the absence of myoepithelial cells leading to deregulated $\beta 1$ integrin action.

We have previously demonstrated that the ratio of bound:free HSPGs in normal epithelial cells was approximately 1:5, in malignant epithelial cells 1:8, and in normal myoepithelial cells 1:1, indicating that there may be a lack of binding sites for the HSPGs in the cell layer of epithelial cells, particularly in the breast carcinoma cell line MCF-7 [63]. The glycosaminoglycan chains of HSPG are the major storage site for many growth factors [64, 65]. Laminin may have a role in binding either two molecules of basement membrane HSPG (perlecan) into the basement membrane and/or as a "bridge" between one molecule of cell surface HSPG (syndecan) and one molecule of perlecan. Whichever role laminin performs, aberrant secretion and/or assembly of the $\alpha 1$ and $\alpha 2$ chains in the MCF-7 cell line or in the luminal epithelial cells in the absence of myoepithelial cells will result in reduced HSPG binding sites and consequently more unbound HSPG. Unbound HSPG is potentially more susceptible to proteolysis than bound HSPG and thus to the release of stored growth factors by the matrix metalloproteinases present in malignant breast tissue such as stromelysin-3 [66] and matrilysin [67]. Alternatively free HSPG-bound growth factor may become more bioavailable to the luminal epithelial cells. HSPG-bound FGF1 has been shown to be one hundredfold more mitogenic than heparin-bound FGF1 [68] and may have a role as a motogen and a mitogen as well as resulting in lack of polarity in transformed mammary epithelial cells [67]. We have shown [70] that an early event in breast neoplasia may be the protease release of FGF-1 from storage sites on adjacent normal ducts and its accumu-

lation in the stroma surrounding the malignant epithelial cells.

We therefore propose that one of the potential functions of the breast myoepithelial cells is the synthesis, secretion, and deposition of laminin $\alpha$ chains, thus enabling the adjacent luminal epithelial cells to have access to their polarizing capabilities and also to bind heparan sulfate proteoglycans into the basement membrane and thus sequester growth factors.

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